HCV-unrelated cryoglobulinaemic vasculitis: the results of a prospective observational study by the Italian Group for the Study of Cryoglobulinaemias (GISC)

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ABSTRACT

Objective. *To investigate the clinical and laboratory patterns of HCV-unrelated CV, and the factors influencing its outcome.*

Methods. *Prospective study of all anti-HCV and HCV-RNA negative patients with CV have been observed since January 2004 in 17 centres participating in the Italian Group for the Study of Cryoglobulinaemias (GISC).*

Results. *175 enrolled were followed up for 677 person-years. The associated conditions were primary Sjögren's syndrome (21.1%), SLE (10.9%), other autoimmune disorders (10.9%), lymphoproliferative diseases (6.8%), solid tumours (2.3%) and HBsAg positivity (8.6%), whereas 69 patients (39.4%) had essential CV. There were significant differences in age (p<0.001), gender (p=0.002), the presence of purpura (p=0.005), arthralgia (p=0.009), liver abnormalities (p<0.001), sicca syndrome (p<0.001), lymphadenopathy (p=0.003), splenomegaly (p=0.002), and rheumatoid factor titres (p<0.001) among these groups. Type II mixed cryoglobulins were present in 96 cases (54.9%) and were independently associated with purpura and fatigue (odds ratio [OR]4.3; 95% confidence interval [CI] 1.8–10.2; p=0.001; and OR2.8; 95%CI 1.3–6.3; p=0.012). Thirty-one patients died during follow-up, a mortality rate of 46/1000 person-years. Older age (for each additional year, adjusted hazard ratio [aHR] 1.13; 95%CI 1.06–1.20; p<0.001), male gender (aHR 3.45; 95%CI 1.27–9.40; p=0.015), type II MCG (aHR 3.31; 95%CI 0.09–1.38; p=0.047) and HBsAg positivity (aHR 7.84; 95%CI 1.20–36.04; p=0.008) were independently associated with greater mortality.*

Conclusion. *HCV-unrelated CV is a multifaceted and often disabling disorder. The associated conditions influence its clinical severity, giving rise to significantly different clinical and laboratory profiles and outcomes.*

Introduction

Mixed cryoglobulins (MCGs) are immune complexes formed by antibodies mainly belonging to the IgG class, and a rheumatoid factor (RF)-like antibody, almost always an IgM (1, 2). According to Brouet *et al.* (3), type II MCG includes monoclonal IgMs, and type III polyclonal IgMs. Circulating MCGs can be detected in association with acute and chronic infections, lymphoproliferative disorders and autoimmune diseases (4-6), and are also a relatively frequent finding in elderly people without any significant associated disorder (7). MCGs can cause the serious disease (4, 8, 9) cryoglobulinaemic vasculitis (CV) and, in most cases, are associated with chronic HCV infection (10-15). HCV-unrelated CV is relatively uncommon (16-18), accounting for 9–15% of all CV cases (17, 19). Autoimmune diseases are a major source of non-infectious CV (20-24). An association with B-cell lymphomas has been clearly shown (4) (and also confirmed in HCV-negative cases) (17), and cryoglobulins can also be detected in some patients with solid cancers (25). Hepatitis B virus (HBV) is probably the cause of some cases of CV (26, 27), and a recent study has attributed some sporadic cases to other viral, bacterial and parasitic diseases (27). Significant MCG levels have also been found as late as six months after the onset of the symptoms of acute Chikungu-

nya virus infection (28). However, the real significance of cryoglobulinaemias in the course of infections other than HCV infection, and their propensity to evolve into full-blown chronic CV syndromes, are still debated. Essential CV is defined as the cases not associated with any known causative disease capable of inducing cryoglobulin production (5).

Many aspects of the natural history and outcomes of HCV-unrelated CV have not yet been investigated, and all of the available come from retrospective studies and case reports. The aim of this study was to recruit and longitudinally follow up patients presenting HCV-unrelated CV, and this report describes the clinical and laboratory characteristics of CV in patients with each of the associated conditions, and the causes and predictive factors of death in the study cohort.

Methods

The participating centres enrolled all the patients with HCV-negative MCG who were being followed on 1 January 2004 (prevalent cases) or were diagnosed thereafter (incident cases). The patients' anonymous data were collected at the time of enrolment and every 12 months during the follow-up period. The clinical manifestations to be reported included purpura, fatigue (defined as weakness even at rest and worsened by light exertion), arthralgia (lasting for at least three months or present in the form of two or more episodes per year), peripheral neuropathy (confirmed by electrophysiological tests), Raynaud's phenomenon, lower limb ulcers, chronic urticaria or other cutaneous manifestations, renal abnormalities (defined as proteinuria and/or serum creatinine levels ≥1.5mg/dL, with or without histological evidence of glomerulonephritis), liver abnormalities (defined as at least three consecutive determinations of abnormal ALT levels and/or ultrasound evidence of fibrosis or steatosis of the liver or ultrasonographically confirmed hepatomegaly), ultrasonographically confirmed splenomegaly, lymphadenopathy involving one or more lymph node groups for at least three months, hyper-viscosity syndrome (evidence of one or more of headache, confusion, blurred vision, visual and hearing loss, and epistaxis with instrumental evidence of increased blood viscosity), sicca syndrome (dry eyes and mouth for at least three consecutive months), hypertension (diastolic blood pressure >90 mmHg for at least six months), and lung abnormalities (asthma or chronic obstructive pulmonary disease [COPD] or pulmonary emphysema).

The laboratory tests requested upon enrolment and once a year during the follow-up were the determination of C3, C4 and rheumatoid factor (RF), serum protein electrophoresis, serum creatinine, AST, ALT, proteinuria, HbsAg, anti-HCV and HCV RNA. The cryoglobulin determinations and measurements were made in accordance with the GISC protocol (29). The cryoglobulins were immunochemically characterised by means of immunoelectrophoresis (29, 30).

The inclusion criteria were cryoglobulin positivity (cryocrit ≥0.5%); at least one episode of palpable purpura, or, alternatively, arthralgia and fatigue with at least one of peripheral neuropathy, Raynaud's phenomenon, lower limb ulcers or nephropathy, and C4 ≤8 mg/dL during the year before enrolment; and negativity for HCV antibodies and HCV RNA at the time of enrolment.

The exclusion criteria were type I cryoglobulinaemia, patients who had cleared HCV RNA after antiviral therapy but maintained circulating cryoglobulins, and those transient cryoglobulinaemias associated with acute infections.

CV-associated autoimmune diseases were defined on the basis of the international criteria (31-35), and grouped as CV associated with primary Sjögren's syndrome (PSS), systemic lupus erythematosus (SLE) or other autoimmune diseases (AID); the patients with non-Hodgkin lymphoma (NHL), other lymphoproliferative disorders or monoclonal gammopathy of uncertain significance (MGUS) were grouped as having lymphoproliferative diseases (LPD); the patients who presented solid tumours six months before or at the time of the first evidence of CV were included in a separate group (ST), as were those who were HBsAg positive at the time of enrolment (HBCV). The cases of CV that were not associated with any diseases known to be a cause of cryoglobulin production were defined as essential cryoglobulinaemias (ECV).

The data were censored as of 31 August 2015, and the patients were considered to be in follow-up until the date of the last available examination or the date of death. The predictability of different outcomes was compared in relation to the associated disorder and the type of MCG found at enrolment. Given the broad spectrum of disorders associated with cryoglobulinaemia and the variety of the treatments administered, the prescribed therapies were not included in the present analyses.

The Mann-Whitney or Kruskal-Wallis non-parametric test and Pearson's chi-squared test (or Fisher's exact test, when necessary) were respectively used to compare the continuous and categorical baseline variables of the patients in the groups of interest. In the longitudinal study, C4 values were compared using Wilcoxon's test for paired samples. The risk factors correlated with the type of cryoglobulinaemia and the variables that independently correlated with the risk of being no longer positive for MCG after 12 months were analysed using logistic regression multivariate models. The survival analyses were made using the Kaplan-Meier method. The long-rank test was used to compare the survival curves between groups. The baseline clinical predictors of survival were assessed using a multivariable Cox proportional hazard regression model. All of the analyses were made using SAS 9.3 (SAS Institute, Cary, NC).

Results

Between 1 January 2004 and 30 June 2013, a total of 246 cases were reported by the 17 participating centre, but 71 were excluded because the did not meet the inclusion criteria or the data were incomplete. The analysis were therefore based on 175 patients (37 males and 138 females), the last of whom was enrolled on 20 May 2013: 101 prevalent cases as of 1 January 2004 (18

Table I. Baseline demographic, clinical and laboratory data relating to patients with HCV-unrelated CV (by associated disorder) or essential CV.

Baseline characteristics	Total n=175	PSS n=37	SLE n=19	AID n=19	LPD n=12	ST n=4	HBCV n=15	ECV n=69	p^*
Females [n (%)]	138 (78.9)	35 (94.6)	19 (100.0)	17 (89.5)	8 (66.7)	3 (75.0)	10 (66.7)	46 (66.7)	0.002
Age (years) [median (IQR)]	66 (55-74)	67 (61-73)	46 (37-62)	60 (46-69)	77 (67-78)	75 (70-78)	63 (55-69)	68 (56-74)	<0.001
Incident cases [n (%)] [§]	74 (42.3)	16 (43.2)	3 (15.8)	6 (31.6)	3 (25.0)	4 (100.0)	4 (26.7)	38 (55.1)	0.004
Purpura [n (%)]	121 (69.1)	29 (78.4)	7 (36.8)	12 (63.2)	10 (83.3)	4 (100.0)	14 (93.3)	45 (65.2)	0.005
Arthralgia [n (%)]	140 (80.0)	30 (81.1)	19 (100.0)	17 (89.5)	7 (58.3)	4 (100.0)	8 (53.3)	55 (79.7)	0.009
Fatigue [n (%)]	123 (70.3)	25 (67.6)	12 (63.2)	15 (78.9)	8 (66.7)	3 (75.0)	10 (66.7)	50 (72.5)	0.949
MF triad [n (%)]	68 (38.9)	15 (40.5)	4 (21.1)	8 (42.1)	4 (33.3)	3 (75.0)	7 (46.7)	27 (39.1)	0.493
Lower limb ulcers [n (%)]	31 (17.7)	8 (21.6)	3 (15.8)	4 (21.1)	4 (33.3)	2 (50.0)	1 (6.7)	9 (13.0)	0.234
Peripheral neuropathy [n (%)]	85 (48.6)	22 (59.5)	7 (36.8)	7 (36.8)	6 (50.0)	3 (75.0)	6 (40.0)	34 (49.3)	0.490
Renal abnormalities [n (%)]	46 (26.3)	13 (35.1)	8 (42.1)	6 (31.6)	3 (25.0)	2 (50.0)	4 (26.7)	10 (14.5)	0.075
Hyperviscosity syndrome [n (%)]	4 (2.3)	1 (2.7)	1 (5.3)	1 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5)	0.999
Raynaud's phenomenon [n (%)]	46 (26.3)	10 (27.0)	9 (47.4)	6 (31.6)	3 (25.0)	0 (0.0)	0 (0.0)	18 (26.1)	0.060
Chronic urticaria or other cutaneous manifestations[¶] [n (%)]	30 (17.1)	7 (18.9)	7 (36.8)	3 (15.8)	1 (8.3)	0 (0.0)	1 (6.7)	11 (15.9)	0.360
Liver abnormalities [n (%)]	41 (23.4)	4 (10.8)	2 (10.5)	2 (10.5)	3 (25.0)	1 (25.0)	12 (80.0)	17 (24.6)	<0.001
Lung abnormalities [n (%)]	26 (14.9)	7 (18.9)	2 (10.5)	4 (21.1)	0 (0.0)	1 (25.0)	3 (20.0)	9 (13.0)	0.552
Sicca syndrome [n (%)]	68 (38.9)	32 (86.5)	8 (42.1)	10 (52.6)	3 (25.0)	0 (0.0)	3 (20.0)	12 (17.4)	<0.001
Hypertension [n (%)]	66 (37.7)	17 (45.9)	3 (15.8)	8 (42.1)	7 (58.3)	2 (50.0)	7 (46.7)	22 (31.9)	0.171
Lymphadenopathy [n (%)]	34 (19.4)	13 (35.1)	6 (31.6)	2 (10.5)	3 (25.0)	2 (50.0)	3 (20.0)	5 (7.2)	0.003
Splenomegaly [n (%)]	27 (15.4)	3 (8.1)	1 (5.3)	3 (15.8)	5 (41.7)	2 (50.0)	6 (40.0)	7 (10.1)	0.002
C4 (mg/dL) [median (IQR)]	4 (2-12)	4 (2-11)	4 (3-10)	8 (1-14)	2 (1-5)	7 (4-12)	2 (1-8)	5 (2-15)	0.544
RF (kU/L) [median (IQR)]	109 (20-372)	239 (90-504)	15 (9-19)	20 (5-58)	341 (106-589)	603 (309-1433)	220 (111-2935)	101 (22-238)	0.001
Cryocrit (%) [median (IQR)]	2.0 (0.5-4.0)	2.0 (0.9-7.0)	1.0 (1.0-2.0)	0.5 (0.5-3.1)	5.5 (3.3-6.5)	6.0 (1.6-12.0)	2.0 (2.0-17.0)	1.2 (0.5-3.0)	0.061
Type of MCG [n (%)]									0.134
Type II	96 (54.9)	24 (64.9)	6 (31.6)	8 (42.1)	9 (75.0)	2 (50.0)	10 (66.7)	37 (53.6)	
Type III	79 (45.1)	13 (35.1)	13 (68.4)	11 (57.9)	3 (25.0)	2 (50.0)	5 (33.3)	32 (46.4)	

*p-values are for χ^2 or Fisher's exact test and Kruskal-Wallis test when comparing all groups in the analysis of each category. [¶] Other than palpable purpura and ulcers.
[§] Diagnosed after 1 January 2004.
PSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; AID: other auto-immune disorders; LPD: lymphoproliferative diseases. ST: solid tumours; HBCV: HBsAg positive cryoglobulinaemic vasculitis; ECV: essential cryoglobulinaemic vasculitis.

males and 83 females) and 74 incident cases (19 males and 55 females). The associated diseases were PSS in 37 cases (21.1%), SLE in 19 (10.9%), AID in 19 (10.9%, including five patients with rheumatoid arthritis, four with mixed connective tissue disease, four with systemic sclerosis, and six with other AIDs), lymphoproliferative diseases in 12 cases (6.8%, including six patients with NHL, two with Waldenström's disease, and four with MGUS), solid tumours in four patients (2.3%, including two follicular thyroid carcinomas, one lung cancer and one liver cancer). HBsAg was positive in 15 patients (8.6%) without any concomitant disease associated with cryoglobulin production. No underlying disease was found in 69 patients (39.4%) who were consequently classified as having ECV.

Women were more prevalent in all of the groups, but their frequency was significantly different, with the highest percentage having autoimmune disease-related CV (PSS, SLE and AID). In particular, 100% of the SLE cases were women, who were also significantly younger than the others (Table I). The prevalence of purpura in the cohort as a whole was 69.1%, with statistically significant differences among the groups. The lowest prevalence was in the patients with SLE, who also showed a significant correlation between purpura and sicca syndrome (p=0.048). There were significant between-group differences in the distribution of arthralgia, with the highest prevalence in the SLE group, which showed the lowest prevalence of the Meltzer and Franklin triad. There were also significant differences in the distribution of liver abnormalities (more frequent in the patients with HBCV), sicca syndrome (more frequent in the patients with PSS), lymphadenopathy (less prevalent in the patients with ECV and AID), and splenomegaly (less frequent in the patients with PSS, SLE and ECV).

The median C4 level in cohort as a whole was 4 mg/dL, (IQR 2-12) without any significant between-group differences, whereas the median RF titres were significantly different, being lowest in the SLE and AID groups. The median cryocrit value was 2% (IQR 0.50–4%) with non-significant variations among the groups.

Type II cryoglobulins were found in 96 cases (54.9%), without any significant between-group differences. The patients with type II MCG more frequently had the Meltzer and Franklin triad, purpura, asthenia, ulcers and peripheral neuropathy, lower C4 levels,

and higher cryocrit concentrations than those with type III MCG (Table II).

On the contrary, arthralgia was more frequent in the patients with type III MCG. Purpura (odds ratio [OR] 4.3; 95% confidence Interval [CI] 1.8–10.2; p=0.001) and fatigue (OR 2.8; 95%CI 1.3–6.3; p=0.012) were independently associated with type II MCG, whereas arthralgia was negatively related (OR 0.3; 95% CI 0.1–0.8; p=0.016).

Twelve months after enrolment, 23.4% of the patients (36/154) were MCG negative. The predictors of becoming MCG negative during follow-up were a longer time since the first diagnosis of CV, and the presence of cutaneous manifestations, liver abnormalities, lymphadenopathy and type III cryoglobulins at enrolment (Table III).

In the patients who became MCG negative, the median C4 level significantly increased from 4mg/dL (IQR 1–9) at baseline to 9 mg/dL (IQR 3–17) at the time of the negative cryoglobulin test (p<0.001). Becoming MCG negative during follow-up independently correlated with the time since the diagnosis of CV (OR 1.1 for each additional year; 95%CI 1.0–1.2; p=0.002) and the absence of fatigue at the time of enrolment (OR 0.3; 95%CI 0.1–0.9; p=0.033). MCG became negative during the first year of follow-up in 43.8% of the patients with SLE and in 38.5% of those of HBCV, but in only 13.7% of those with ECV and 16.7% of those with AID; in the other three groups, about 25% of the patients became MCG negative. The percentage of patients who became MCG negative was significantly lower in the ECV group than in the other groups taken together (13.7% *vs*. 29.1%, p=0.029). Fifty-seven (35.2%) of the 162 patients for whom this information was available became MCG negative at some time during the follow-up. The time from the first diagnosis of CV was longer in these patients than in those who remained MCG positive (p<0.001), they were more frequently women (p=0.005), and they more frequently had Raynaud's phenomenon (p=0.008), cutaneous manifestations other than lower limb ulcers (p=0.025), liver abnormalities (p=0.029), and lymphadenopathy (p=0.001).

Table II. Baseline characteristics of 175 patients with HCV-unrelated CV by type of MCG.

Characteristics	Type II n = 96	Type III n = 79	p^*
Female [n (%)]	72 (75.0)	66 (83.5)	0.168
Age (years) [median (IQR)]	66 (57-74)	64 (47-74)	0.419
Incident cases [n (%)] §	38 (39.6)	36 (45.6)	0.425
Purpura [n (%)]	82 (85.4)	39 (49.4)	<0.001
Arthralgia [n (%)]	69 (71.9)	71 (89.9)	0.003
Fatigue [n (%)]	75 (78.1)	48 (60.8)	0.012
MF triad [n (%)]	46 (47.9)	22 (27.8)	0.007
Lower limb ulcers [n (%)]	23 (24.0)	8 (10.1)	0.017
Peripheral neuropathy [n (%)]	55 (57.3)	30 (38.0)	0.011
Renal abnormalities [n (%)]	26 (27.1)	20 (25.3)	0.792
Hyperviscosity syndrome [n (%)]	3 (3.1)	1 (1.3)	0.628
Raynaud's phenomenon [n (%)]	25 (26.0)	21 (26.6)	0.936
Chronic urticaria or other cutaneous manifestations¶ [n (%)]	12 (12.5)	18 (22.8)	0.072
Liver abnormalities [n (%)]	26 (27.1)	15 (19.0)	0.208
Lung abnormalities [n (%)]	13 (13.5)	13 (16.5)	0.590
Sicca syndrome [n (%)]	38 (39.6)	30 (38.0)	0.828
Hypertension [n (%)]	37 (38.5)	29 (36.7)	0.803
Lymphadenopathy [n (%)]	17 (17.7)	17 (21.5)	0.526
Splenomegaly [n (%)]	19 (19.8)	8 (10.1)	0.078
C4 (mg/dL) [median (IQR)]	3 (1-8)	7 (3-14)	0.009
RF (kU/L) [median (IQR)]	135 (30-495)	49 (19-278)	0.141
Cryocrit (%) [median (IQR)]	3.0 (1.0-9.0)	1.0 (0.5-2.0)	0.001

*p-values are for χ^2 or Fisher's exact test and Mann-Whitney test. § Diagnosed after 1 January 2004. ¶ Other than palpable purpura and ulcers.

The patients were followed-up for a total of 677 person-years (p-y). The median follow-up time was 37 months (IQR 12–67), and was significantly different between the groups, being significantly shorter in the LPD group. Seven patients died and four were lost to follow-up within 12 months of enrolment.

A total of 31 patients (10 males and 21 females) died during the follow-up, a mortality rate of 4.6 per 100 p-y. The median age at death was 75 years (IQR 67–83). The causes of deaths are described in Table IV.

Figure 1 (panel A) shows the time-dependent survival probability in the cohort as a whole. The Kaplan-Meier survival estimates (95% CI) were 0.92 (0.87–0.96) at the end of the first year of follow-up, 0.82 (0.74–0.89) at the end of the fourth year, and 0.66 (0.54–0.79) at the end of the eighth year. The probability of survival was significantly lower among the males (Fig. 1, panel B, p=0.004) and lower (of borderline statistical significance) among the patients with type II MCG at baseline (Fig. 1 panel C, p=0.072). The patients with purpura had a lower survival probability than those without (Fig. 1 panel D, p=0.043).

Mortality was lower in the SLE and AID groups taken together than in the other groups (Fig. 2, p=0.004), and higher during the first two years of follow-up in the HBCV group.

Univariate analysis showed that male gender and each additional year of age were correlates of a higher risk of death, whereas each additional year from CV diagnosis and having SLE/AID were protective factors (Table V). Multivariate analysis confirmed that an older age and male gender were independently associated with greater mortality, and showed that having type II MCG and belonging to the HBCV group were also independent correlates of greater mortality.

Discussion

The findings of this study confirm that HCV-unrelated CV is not a benign disease. One of the aims of the study was

Table III. Baseline characteristics of patients who did or did not continue producing mixed cryoglobulins (MCG) 12 months after enrolment.

Characteristics	MCG		*p**
	Permanently positive n=118	Negativised n=36	
Female [n (%)]	89 (75.4)	32 (88.9)	0.085
Age (years) [median (IQR)]	64 (55-74)	66 (55-75)	0.823
Years since diagnosis [median (IQR)]	5 (2-9)	13 (4-17)	<0.001
Purpura [n (%)]	80 (67.8)	24 (66.7)	0.899
Arthralgia [n (%)]	95 (80.5)	30 (83.3)	0.704
Fatigue [n (%)]	90 (76.3)	22 (61.1)	0.074
MF triad [n (%)]	49 (41.5)	12 (33.3)	0.379
Lower limb ulcers [n (%)]	22 (18.6)	5 (13.9)	0.511
Peripheral neuropathy [n (%)]	55 (46.6)	16 (44.4)	0.820
Renal abnormalities [n (%)]	33 (28.0)	11 (30.6)	0.763
Hyperviscosity syndrome [n (%)]	4 (3.4)	0 (0.0)	0.574
Raynaud's phenomenon [n (%)]	28 (23.7)	14 (38.9)	0.074
Chronic urticaria or other cutaneous manifestations[¶] [n (%)]	16 (13.6)	11 (30.6)	0.019
Liver abnormalities [n (%)]	23 (19.5)	14 (38.9)	0.017
Lung abnormalities [n (%)]	14 (11.9)	4 (11.1)	0.902
Sicca syndrome [n (%)]	46 (39.0)	14 (38.9)	0.992
Hypertension [n (%)]	41 (34.7)	16 (44.4)	0.291
Lymphadenopathy [n (%)]	16 (13.6)	15 (41.7)	<0.001
Splenomegaly [n (%)]	19 (16.1)	5 (13.9)	0.749
C4 (mg/dL) [median (IQR)]	5 (2-15)	4 (1-9)	0.091
RF (kU/L) [median (IQR)]	119 (21-386)	114 (22-546)	0.743
Cryocrit (%) [median (IQR)]	2.0 (0.5-5.0)	1.8 (0.9-4.5)	0.952
Type of MCG [n (%)]			0.015
Type II	73 (61.9)	14 (38.9)	
Type III	45 (38.1)	22 (61.1)	
Associated disorder [n (%)]			0.133
PSS	24 (20.3)	9 (25.0)	
SLE	9 (7.6)	7 (19.4)	
AID	15 (12.7)	3 (8.3)	
LPD	9 (7.6)	3 (8.3)	
ST	3 (2.5)	1 (2.8)	
HBCV	8 (6.8)	5 (13.9)	
ECV	50 (42.4)	8 (22.2)	

p-values are for χ^2 or Fisher's exact test and Mann-Whitney test.
[¶]Other than palpable purpura and ulcers.

to identify the disorders that are more likely to be responsible for chronic CV and differentiate them from transient cryoglobulinaemia of no pathological significance. The overlapping of some of the signs and symptoms of CV with those characterising the associated diseases may be a major confounding factor. After carefully examining the clinical and laboratory data, about 30% of the cases proposed for enrolment in the study were excluded, mainly because they presented MCG accompanied by symptoms compatible with the underlying disease but not sufficient for a diagnosis of CV on the basis of our inclusion criteria. When the study was designed in 2003, there were no validated CV classification criteria, but those recently proposed by the GISC (36, 37) perform well also in the case of HCV-unrelated CV (38) (albeit with slightly lower specificity), and are currently under evaluation in this case series.

As in the case of HCV-associated cryoglobulinaemias (15, 16, 39), CV is significantly more frequent in females aged >60 years. This finding is similar to that described by Foessel *et al*. (18) in their study of type II MCGs, and that found in a larger French study (20).

Palpable purpura was observed in 75% of the cases of Terrier *et al*. (40) and in 69.1% of our patients, and in 78% and 85.4% of the patients with type II MCG. In our series, it was also one of the factors independently associated with type II MCG, although it does not seem to be a predictor of MCG persistence as its prevalence was similar in the patients who became MCG-negative during follow-up and in those who continued to produce them. On the other hand, the loss of MCG production was predicted by the time from the first MCG-positive test, thus suggesting that longer treatment exposure may influence their clearance.

Peripheral neuropathy (PN) is frequently part of CV syndrome, and sometimes the first sign of cryoglobulinaemia (5, 41, 42). In an Italian study, 52% of the patients with HCV related CV had PN (39), which is similar to the approximately 49% prevalence in our study. Taken together, these findings confirm that PN is frequent in patients with CV regardless of its cause. Moreover, it was significantly more frequent in patients with type II MCG.

Lung disorders were frequently seen in our case series, mainly in elderly patients and without a clear-cut relationship with CV. Fewer than 3% of the patients were affected by hyperviscosity syndrome (about the same percentage as that reported among HCV-related cases) (15, 43), thus confirming that it is uncommon in mixed cryoglobulinaemias regardless of their cause.

Sicca syndrome is frequently observed in patients with HCV infection (44, 45), and in those with HCV-associated CVs (46). In our series, it was not only frequent in patients with autoimmune disorders, but also relatively frequent in those with HBCV and ECV. The recent demonstration of salivary gland epithelial cell infection by human T-lymphtropic virus-1 (HTLV-1) (47), and previous data concerning the presence of HTLV-1 infection in patients with ECV (48) suggest that the role of this

Table IV. Causes of death in 31 patients with HCV-unrelated CV.

Associated disorder	Observed deaths		Percentage of total number of observed deaths	Notes
	No.	%		
PSS	9/37	24.3	29.0 and	Four deaths were caused by NHL occurring after enrolment, three cardiac-related deaths, one due to renal insufficiency and rituximab-induced severe thrombocytopenia. The cause of death was unknown in one case.
SLE	1/19	5.2	3.2	Caused by gastric bleeding
AID	1/19	5.2	3.2	Caused by suicide
LPD	0/12	-	-	Nine patients were lost to follow-up. The other three patients (with Waldenström disease, gastric MALT lymphoma and MGUS respectively) were still alive at the time of data analysis.
ST	1/4	25.0	3.2 to	One patient with primary liver cancer died 28 months after diagnosis. This patient was repeatedly HBsAg and HCV RNA negative, but positive for anti-HBc antibodies. Two patients with follicular thyroid carcinomas were transferred to oncological centres and lost follow-up. The last patient had a diagnosis of lung carcinoma in 2004 and was still alive in 2011, when she was lost to follow-up.
HBCV	6/15	40.0	19.4	The causes of death were different in each patient and included NHL, hepatitis B exacerbation, acute pneumonia, renal insufficiency, cachexia in Wernike's encephalopathy, and cardiac arrest in senile marasmus.
ECV	13/69	18.8	42.0	There were three cardiac deaths, three cases of pneumonia (one after treatment with rituximab), one NHL, one lung carcinoma, one death due to gastric bleeding, and one case of stroke. The cause of death was unknown in three cases.

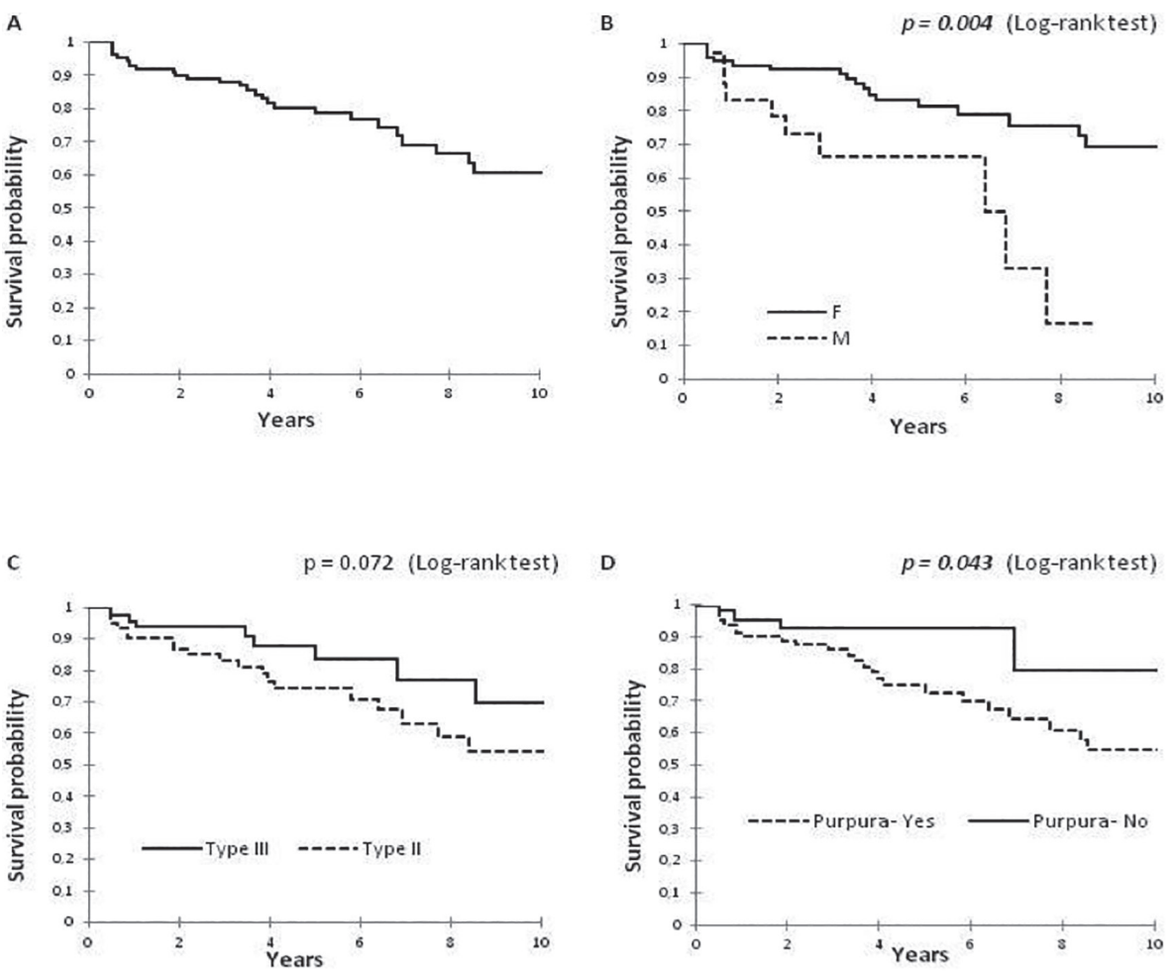

Fig. 1. Time-dependent cumulative probability of survival during follow-up: overall probability (panel A); males *vs*. females (panel B); type II *vs*. type III cryoglobulinaemia (panel C)); patients with *vs*. patients without purpura (panel D).

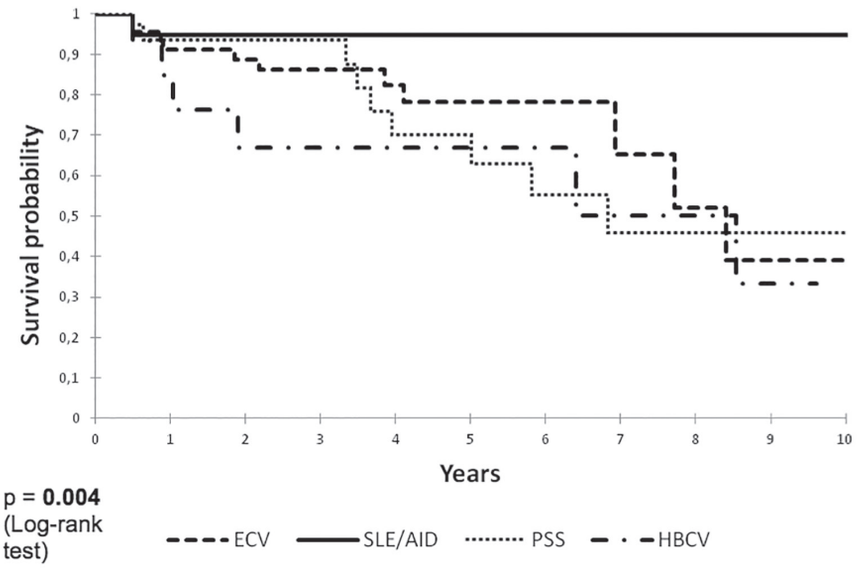

Fig. 2. Time-dependent cumulative probability of survival during follow-up by CV-associated disorder.

Table V. Variables associated with an increased risk of dying during follow-up (Cox model).

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age (for each additional year)	1.06	1.03-1.09	**<0.001**	1.13	1.06-1.20	**<0.001**
Male gender	2.97	1.37-6.45	**0.006**	3.45	1.27-9.40	**0.015**
Years since diagnosis (for each additional year)	0.94	0.88-0.99	**0.036**	0.93	0.84-1.02	0.118
Purpura	2.82	0.98-8.07	0.05	0.35	0.09-1.38	0.134
Type II cryoglobulinaemia	2.00	0.92-4.35	0.079	3.31	1.02-10.78	**0.047**
ECV	1	-	-	1	-	-
SLE/AID	0.13	0.03-0.60	**0.009**	0,73	0.08-6.99	0.782
PSS	1.04	0.44-2.46	0.924	1,83	0.56-5.93	0.314
HBCV	1.60	0.60-4.26	0.349	7,84	1.70-36.04	**0.008**
MCG negative after 12 months	0.51	0.17-1.53	0.228	0.39	0.10-1.61	0.195

virus as a possible cause of EMC with sicca syndrome should be reassessed.

In comparison with HCV-related CV (39), the historical GISC series (9). and the French HCV-negative series (20), the median cryocrit levels observed in our cohort at the time of enrolment was relatively low. However, almost all of the patients included in 2004 were on treatment with immunosuppressive drugs or steroids and, in the majority of cases, their cryocrit levels at enrolment were considerably lower than those previously observed.

It has been hypothesised that cryoprecipitating RF production proceeds from poly-clonality (*i.e.* type III cryoglobulins) to oligo-clonality, and finally mono-clonality (13, 49). In patients with HCV-related CV, the development of type II MCG is considered a hallmark of CV (13, 50, 51), but whether or not the type III MCG produced in association with certain diseases progress towards monoclonality is still unknown. Type III MCGs were more frequent in our cases than in the French series (40), in which haematologic and connective tissue diseases accounted for respectively 30% and 22% of the cases whereas, after excluding the HbsAg-positive patient, LPD and connective tissue diseases accounted for 7.5% and 46.9% of the cases in our series. It is conceivable that this case mix was due to the characteristics of the GISC centres, which are mainly rheumatology centres.

The Kaplan-Meier survival estimates in our cohort were quite similar to those of Terrier *et al*., (40) and an older age and male gender were independently associated with a poor prognosis in both studies. This finding strongly suggests that, although less frequent, CV may be a more serious disease in males than in females. Type II MCG, which is an independent risk factor of increased mortality, was further confirmed as a marker of advanced disease.

MCGs have been reported in 10–15% of patients with PSS (17, 52), which our study confirms to be a leading cause of HCV-unrelated CV. Overt purpura was present in 78% of our PSS patients, 64% of whom had type II MCGs that persisted over time in more than 70% of cases. During the study period, four PSS patients died because of NHL. It is known that PSS increases the risk of developing B-cell NHL (23, 53, 54), and PSS patients show a polyclonal pattern of B cell expansion in bone marrow more frequently than patients with HCV-related cryoglobulinaemia (46).

The clinical and laboratory profile of SLE-related CV was significantly different from those of the other groups. The cessation of MCG production during the first year after enrolment may be due to the fluctuating nature of autoantibody production in SLE, but also to SLE treatment. However, how many SLE patients with MCGs have real CV and the role of MCGs in the natural history of SLE are still unclear.

In the AID group, cryocrit and RF concentrations tended to be higher in the patients with RA or mixed connective tissue disease than in the others. All of the patients with systemic sclerosis had Raynaud's phenomenon, sicca syndrome and type III MCGs, and lower limb ulcers were recorded in 3 cases, but purpura was recorded in only one patient who had the lowest median RF titre in the AID group. Taken together, these findings suggest that the inclusion criteria adopted in this study may be unable to identify real CV in patients with autoimmune diseases.

Waldenström's disease is more frequently described as a cause of type I cryoglobulinaemia, but the two cases found in our study confirm our previous observation about its possible involvement in MCG (4, 9).

The association of CV with solid tumours seems to be uncommon, as has also been suggested by other studies (25). Purpura was present in all four of our cases, three of whom continued to produce MCGs during the follow-up, thus preventing us from excluding the possibility that CV may be a paraneoplastic syndrome in some cases. The positivity of anti-HBc antibodies in a patient with CV who died of primary liver cancer raises the question of the role of occult HBV infection in causing both disorders.

The reported percentage of CV caused by HBV ranges from 0.5% to 3% (16, 17). HBsAg was positive in 5.8% of the 717 patients in the GISC series for whom this information was available.[9] After excluding the patients with evidence of liver disease, its prevalence was similar to that of blood donors living in the same area (55). These observations originally led us to conclude that HBV does not play a pivotal role in causing CV and that it may play an aetiological role in just a small minority of cases. Accordingly, Terrier *et al*. reported a total of only ten HBV-related cases (27), even if another study described 48 patients (56) (ten of whom were part of a group of North African Jewish women with Raynaud's syndrome) (57) and eight further cases were found in a retrospective survey carried out in China (58). However, the finding of only one HBV-related CV in the GISC population-based survey[7] further suggests that it is rare. No study has yet investigated what percentage of patients with chronic HBV infection may develop CV. It has been recently suggested that, like HCV, HBV may cause MGC by protracting the antigenic stimulation of VH1-69-expressing B cells (59). Our study adds 15 unpublished cases to the relatively small number of known patients with HBV-related CV, 12 of whom showed significant liver abnormalities at the time of enrolment, thus suggesting active liver disease in most cases. Moreover, 14 of the 15 patients had purpura and 10 had type II MCG, and multivariate analysis showed that they were at increased risk of death. Given the small number of cases and the different timing of antiviral treatment, it was not possible to state whether antiviral drugs influenced their outcomes or the disappearance of MCG during the follow-up. Interestingly, excluding these HBV-associated cases, none of the infection-related cases of MCG observed during the study period fulfilled our inclusion criteria.

True cases of ECV are probably rare as just one patient with overt CV unrelated to any other disease was found in the GISC population–based study (7). Despite this, ECV was the largest group in our study and in two other major series (16, 20), thus accounting for more than 40% of the patients with HCV-unrelated CV. Type III MCG was more represented in our ECV patients (46%) than in the French series (about 14%) (20). However, a number of our ECV cases evolved into type II MCG during the follow-up. The fact that these patients became MCG negative less frequently than the others may be due to the less effective treatment available for ECV.

In conclusion, our findings confirm that CV is a multifaceted and often disabling disorder also in HCV-negative cases. Male patients with purpura and type II MCG experience the worst outcomes, regardless of the underlying disease. Each associated condition influences the clinical severity and stability of MCG production differently over time, and leads to significantly different clinical and laboratory profiles and outcomes. The most divergent groups, SLE and AID, were also those in which the inclusion criteria used in this study had major limitations due to the overlap of some of the symptoms frequently associated with these disorders (*e.g*. Raynaud's phenomenon) and the symptoms of CV. Essential MCGs remain an enigma that the longer follow-up of this cohort might contribute to solve.

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